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THE UNIVERSITY OF ALBERTA

Evidence for Hormonal Control of Dopa Decarboxylase in *Drosophila* from *In Vitro* Translation Studies

by

Gregory P. Kraminsky

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Evidence for Hormonal Control of Dopa Decarboxylase in *Drosophila* from *In Vitro* Translation Studies submitted by Gregory P. Kraminsky in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The mRNA coding for dopa decarboxylase in *Drosophila* has been identified. Poly(A)-RNA from two samples of wild-type larvae of different ages was translated *in vitro* in both the wheat-germ and the mRNA-dependent reticulocyte lysate protein synthesizing systems. In both, a product was synthesized which was immunoprecipitable by anti-DDC IgG and comigrated with purified DDC on SDS-polyacrylamide gels. More immunoprecipitable material was synthesized in response to RNA from late third instar larvae than mid-third instar larvae, suggesting that more DDC mRNA was present at the later stage. This observation correlates well with native enzyme activity levels, which rise rapidly late in the third instar.

The effects of ecdysterone on DDC mRNA production were analyzed using the temperature-sensitive *ecd*^{1} mutant. When third instar *ecd*^{1} larvae are raised to 29°C, the peaks of ecdysterone and DDC activity, which normally occur just prior to pupariation, fail to appear. Poly(A)-RNA from epidermis of these larvae had no detectable mRNA activity, as determined by translation and immunoprecipitation studies. When *ecd*^{1} larvae, previously raised to 29°C are fed on a solution of ecdysterone, DDC activity appears rapidly. These larvae, after eight hours of exposure to the hormone, were shown to contain translatable DDC mRNA. These results were interpreted as evidence that, during normal

development, ecdysterone induces the production of DDC mRNA late in the third instar.

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LIST OF ABBREVIATIONS

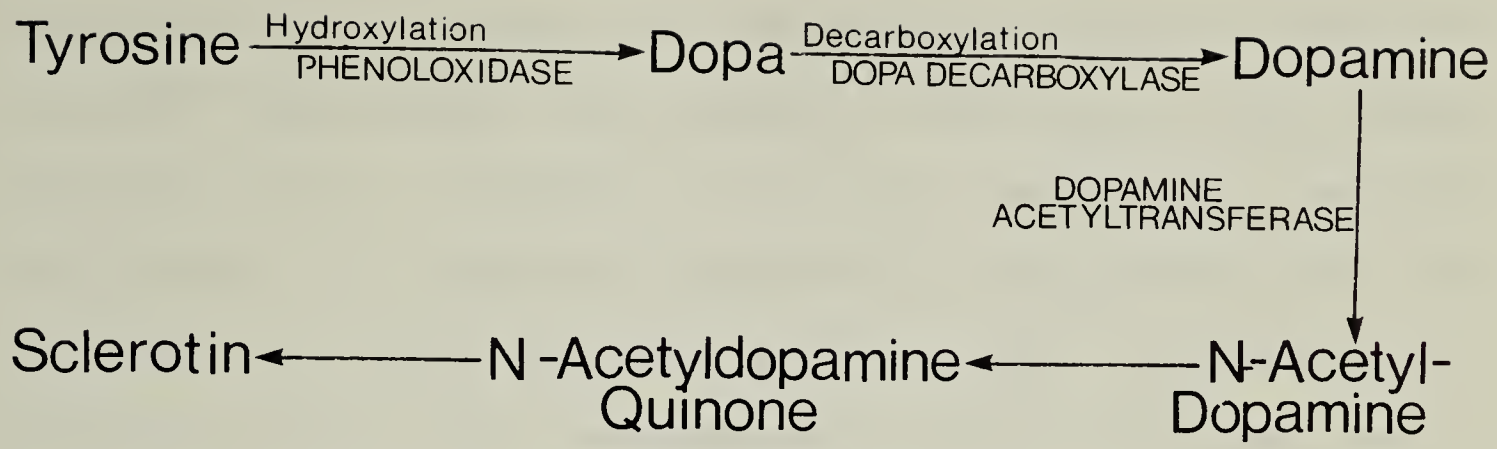
ATP	Adenosine 5'-triphosphate
cDNA	DNA transcribed from messenger RNA
CPM	Counts per minute
C.S.	Canton-Special
DDC	Dopa decarboxylase
EGTA	Ethyleneglycol-bis-(B-amino-ethyl ether) N,N'-tetraacetic acid
GTP	Guanosine 5'-triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
mRNA	Messenger RNA
MW	Molecular weight
PTU	Phenylthiourea
SDS	Sodium dodecyl sulfate
Tris	Iris(Hydroxymethyl)amino methane
SA	Staphylococcus aureus cells

I. INTRODUCTION

The processes of differentiation and development will only be fully understood when the mechanisms of gene regulation have been elucidated. At this time, most of the factors controlling gene activation and regulation are unknown. One approach to the problem has been to study the control of genes whose products can easily be identified by biochemical means. This has made it possible to examine some of the factors which selectively activate specific genes, and it is hoped that these studies will contribute to a general understanding of the formation and maintenance of differentiated states. The regulation of Dipteran dopa decarboxylase may represent a model system for the study of selective gene activation.

A. Physiological Properties of Dopa Decarboxylase

The enzyme dopa decarboxylase (DDC; EC 4.1.1.26) is involved in the hardening and darkening (sclerotization) of insect cuticles. Karlson and his co-workers elucidated much of the biochemistry of the sclerotization process in the blowfly Calliphora erythrocephala (see Karlson and Sekeris, 1966, for review). They found dopa decarboxylase to be one of the key enzymes involved in the production of N-acetyldopamine, the sclerotizing agent. The pathway in late third instar larvae of Calliphora is shown below (from Sekeris and Karlson, 1966).



The appearance of dopa decarboxylase activity in developing insects is both tissue and stage-specific. Studies using Calliphora (Shaaya and Sekeris, 1965), Drosophila (McCaman et al., 1972), and Sarcophaga (Chen and Hodgetts, 1974) indicate two major peaks of DDC activity during postembryonic development. During both these stages, most of the DDC activity can be localized to the epidermal cells (Lunan and Mitchell, 1969). The first peak of enzyme activity occurs late in the third instar, and is responsible for the sclerotization of the puparium. The second occurs at adult eclosion, when adult cuticular structures are being sclerotized. In addition, small peaks of DDC activity have been identified in Drosophila late in embryogenesis, (D. Gietz, pers. comm.), and at both larval molts (M. Estelle, pers. comm.; Marsh and Wright, 1979).

In the mosquito Aedes aegypti, DDC is involved in normal oocyte maturation (Schlaeger and Fuchs, 1974a). It

was shown that following a blood meal, adult females displayed an increase in DDC activity, which accompanied ovarian development and oocyte maturation. The enzyme activity was localized to the developing ovaries. Furthermore, it appeared that DDC was incorporated into the oocytes, as sclerotization of the eggs occurred within several hours after oviposition. Recent studies with a temperature-sensitive mutant of Drosophila suggest a similar role for DDC in this organism (Marsh and Wright, 1979).

B. Role of Ecdysterone in DDC Regulation

Throughout this thesis, the nomenclature described by Goodwin et al. (1978) will be used. The term "ecdysterone" will refer specifically to beta-ecdysone (or 20-hydroxyecdysone)--the active form of the hormone. The prohormone, alpha-ecdysone, is secreted by the prothoracic glands in insects (Chino et al., 1974; King et al., 1974). This inactive form of the hormone is converted to ecdysterone in several insect tissues (King and Siddall, 1974).

Karlson and Sekeris (1962) provided the first evidence for the induction of DDC by the steroid moulting hormone, ecdysterone. When third instar larvae of Calliphora were ligated at the third to fourth segment so as to prevent transport of the hormone to the abdomen, the posterior portion of the larvae failed to pupariate, and contained only low levels of DDC. Injection of alpha-ecdysone into the

abdomens of the ligated larvae resulted in the appearance of DDC activity within 7-10 hours, and subsequent pupariation. Presumably, the alpha-ecdysone was converted to ecdysterone after injection. Later comparisons of ecdysterone and DDC activity levels during development revealed a peak in ecdysterone titre concomitant with the rise in DDC activity at pupariation (Shaaya and Sekeris, 1965). These two observations led Karlson and his co-workers to propose that at pupariation, the appearance of DDC activity is dependent upon a prior increase in the titre of ecdysterone.

Subsequent studies (Sekeris and Karlson,1964) demonstrated that inhibition of either RNA synthesis or protein synthesis at the time of ecdysterone secretion prevented the normal rise in DDC activity, and delayed puparium formation. The authors interpreted these results as additional evidence for the hormonal control of enzyme production, and support for a previously proposed model for hormone action (Karlson, 1963). In this model, the hormone acts directly on the DNA to induce production of a specific messenger RNA, which is then transported to the cytoplasm and translated into the encoded protein. This model was based on work in which the injection of ecdysterone into Chironomus larvae resulted in new "puffs" occurring in the salivary gland chromosomes (Clever and Karlson, 1960). These puffs were believed to be regions of active RNA synthesis.

Since these early experiments, a large body of evidence has accumulated to support the hypothesis that ecdysterone

induces DDC in several insect species. In Drosophila melanogaster, ecdysterone and DDC profiles were found to be similar to those in Calliphora, with coincident high titres occuring at pupariation (Hodgetts et al., 1977). In the mosquito Aedes aegypti the increase in DDC activity in the developing ovaries of blood-fed females can be stimulated by injection of ecdysterone into unfed females (Schlaeger and Fuchs, 1974b). In the fleshfly, Sarcophaga bullata , DDC activity can be induced precociously by injection of ecdysterone into early third instar larvae (Chen and Hodgetts, 1974). In addition, an increase in DDC activity in imaginal wing discs cultured in vitro in a medium supplemented with ecdysterone was observed. Finally, Fragoulis and Sekeris (1975a) were able to show that the injection of ecdysterone into isolated abdomens of Calliphora vicina caused de novo enzyme synthesis, as determined by immunoprecipitation of DDC labelled in vivo.

Since the early suggestion by Karlson that ecdysterone acts to induce transcription of the gene coding for dopa decarboxylase, several attempts to verify this proposal have been made. To date, however, none has shown conclusively that the production of mRNA is the primary response to the hormone. Early work utilized an in vitro protein synthesizing system from rat liver directed by RNA from both early third instar Calliphora larvae ("control") and prepupae ("hormone-induced"). The results of these experiments showed apparent dopa decarboxylase activity in

reactions programmed with "hormone-induced" RNA, but not "control" RNA (Sekeris and Lang, 1964; Karlson and Sekeris, 1966). Since it is unlikely that such an inefficient protein synthesizing system directed by bulk RNA would be able to produce active enzyme *in vitro*, and because no further reports appeared in the literature, it would seem that alternative explanations might be needed to account for these results. More recent experiments using an efficient cell-free translation system and immunoprecipitation procedures have provided more conclusive evidence for the production of DDC *in vitro* (Fragoulis and Sekeris, 1975b). In this work, poly(A)-RNA from 6-7 day old larvae was not able to direct synthesis of immunopreciptable DDC, while that from white prepupae did show DDC mRNA activity. Since ecdysterone levels are low in 6-7 day old larvae, and high in white prepupae, it was concluded that the messenger activity present at that stage was induced by ecdysterone. Two points should be made concerning these experiments.

First, the immunoprecipitable radioactivity (representing DDC) migrated as a single band when electrophoresed in a SDS-polyacrylamide gel, while the native enzyme consists of two non-identical subunits of 46,000 and 50,000 daltons (Fragoulis and Sekeris, 1975c). The authors suggest that both subunits were being synthesized, and the single peak of radioactivity was the result of problems of resolution. Another explanation might be that the enzyme is in fact a homodimer , as it is in

Drosophila (Clark *et al.*,1978), and only one mRNA species was being translated into immunoprecipitable material.

The second, and more important feature of this work is the assumption that, since DDC mRNA is present at pupariation, when ecdysterone titre is high, and not in 6-7 day old larvae, when ecdysterone titre is low, the hormone induces the synthesis of that mRNA. However, the 24-48 hour period between the two developmental stages could be ample time to allow several regulatory steps to occur. Most steroid-induced responses are detected in target tissues within an hour of exposure to the hormone(see below). One of the goals of this research was to attempt to provide more conclusive evidence for the induction of DDC mRNA production by ecdysterone.

C. Steroid Hormone Action

Numerous studies, using a variety of model systems, have suggested a common mode of action for most steroid hormones (O'Malley and Means, 1974). This mechanism involves the binding of the hormone with a specific cytoplasmic receptor such that the complex has an increased affinity for specific sites on the chromosomes. The hormone receptor complexes may bind many chromosomal sites non-specifically, but at certain high-affinity sites (possibly specified by certain non-histone proteins) a structural alteration of the chromatin occurs and transcription is initiated (Yamamoto and Alberts, 1976). The induced RNA transcripts then are

transported to the cytoplasm, where they are translated into structural proteins or regulatory proteins which can further alter the pattern of gene activity in those cells. Most of the discussion presented here deals with the detection of the primary response to hormone exposure.

Toft and Gorski(1966) first identified a cytoplasmic receptor protein that is capable of binding ^{3}H-estradiol, from rat uterine tissue. Since this protein was limited specifically to the steroid target tissues and had a high affinity for ^{3}H-estradiol, it was termed a "receptor" protein. Receptor proteins for many different steroid hormones have now been identified, including glucocorticoid receptors in cultured rat hepatoma cells (Baxter and Tomkins, 1971), progesterone receptors in chick oviduct (Sherman et al., 1970) and ecdysteroid receptors in Drosphila cell cultures (Maroy et al., 1978) and imaginal discs (Yund et al., 1978). It is now generally accepted that hormone-receptor binding is the essential initial event in hormone-mediated responses.

Inhibitor studies have indicated that the primary response to hormone exposure is the synthesis of RNA. Means and Hamilton(1968) demonstrated that the administration of estrogen to ovariectomized rats resulted in a 40% increase in uterine RNA synthesis within 2 minutes. This response could be inhibited by actinomycin D, but not cycloheximide, indicating that protein synthesis is not involved in this response.

Some of the more intriguing studies in elucidating the primary response deal with the ecdysterone-induced puffing in the polytene chromosomes of Drosophila salivary glands (Ashburner et al., 1973). Experiments using isolated salivary glands cultured in vitro have revealed a set of "early" puffs which appear within 5 minutes of ecdysterone addition. After a 3 to 4 hour lag period, during continuous exposure to the hormone, a large number of "late" puffs appear. Induction of the early puffs was found insensitive to inhibitors of protein synthesis, while the appearance of late puffs was not. Furthermore, the normal regression of early puffs was shown to be dependent on protein synthesis. On the basis of these and other experiments, it was suggested that the hormone induces transcription at a few specific sites, and the information encoded by these RNAs in turn induces transcription at many loci (late puffs). In addition, one early gene product seems to be responsible for terminating RNA synthesis in the early puffs.

In further attempts to define the primary response to hormone action, messenger RNAs for specific hormone-induced proteins have been identified. Chan et al. (1973) have detected the appearance of avidin mRNA in chick oviduct 6 hours after progesterone administration. Similarly, Rhoads et al. (1973) have demonstrated the dependence of ovalbumin mRNA activity in oviduct on the injection of estrogen into immature chicks. Lastly, Killewich and Fiegelson (1977) have shown that the precocious induction of tryptophan

2,3-dioxygenase activity by glucocorticoids in rat liver was paralleled by an increase in the corresponding mRNA production.

Further insight into the mechanism of hormone action requires an examination of the factors which determine the specificity of binding of the hormone-receptor complex to particular regions of chromatin in a given target tissue. Techniques now available allow the isolation and amplification of specific eukaryotic gene sequences in bacterial plasmids. The first step involves the identification and isolation of the messenger RNA. This mRNA can be used to construct a synthetic gene or cDNA (Maniatis et al., 1976), or to isolate the natural gene by hybridization to cloned DNA fragments (Grunstein and Hogness, 1975). The cloning of the structural gene and its adjacent sequences could allow the identification and characterization of regulatory sites.

The binding of hormone-receptor complexes to DNA is relatively non-specific, and the large numbers of low affinity binding sites appears to prohibit the detection of a small number of high-affinity sites (Yamamoto and Alberts, 1975). The use of cloned genes obviates this problem, and allows an analysis of the hormone-receptor interaction with specific regulatory sites, providing that they are, as is often assumed, adjacent to structural genes (see Britten and Davidson, 1969).

The specificity of receptor-DNA binding appears to be

mediated by chromosomal proteins, of which the non-histones are the implicated regulatory molecules (see O'Malley et al., 1977, for review). The interaction of chromosomal proteins with specific DNA sequences is probably involved in receptor recognition; sequence analysis of cloned genes and regulatory sites may provide some insight into the nature of this interaction.

D. Purpose of this Research

The availability of well defined genetic systems in Drosophila provides a unique opportunity to study gene regulation at the molecular level. Putative regulatory elements controlling gene activity have been identified (Chovnick et al., 1976). To date, however, dopa decarboxylase is the only genetically characterized enzyme system clearly under steroid hormone control.

Recent studies have focused on the characterization of the DDC structural gene, including its localization (Hodgetts, 1975), and the isolation of mutants affecting enzyme production (Wright et al., 1976). The purification and characterization of the enzyme (Clark et al. 1978) provide the basis for more extensive biochemical investigations of hormone action in this organism.

The recent isolation of a temperature-sensitive mutant of Drosophila incapable of ecdysterone synthesis at the restrictive temperature (Garen et al., 1977), has provided the means for a detailed study of ecdysterone-induced

functions. The mutation, ecd^1, results in the inability of larvae to pupariate when raised to the non-permissive temperature early in the third instar. This was shown to be the result of the absence of the high titre of ecdysterone which normally preceeds puparium formation. Normal development can be restored either by exogenous feeding of ecdysterone, or by returning the larvae to the permissive temperature.

This thesis describes the development of an assay for the dopa decarboxylase messenger RNA--the first step in our attempt to clone the DDC structural gene. This assay has permitted an analysis, using the ecd^1 mutant, of the effects of ecdysterone on DDC mRNA production.

II. MATERIALS AND METHODS

A. Maintenance of Stocks

Drosophila melanogaster stocks used were the Canton-S wild-type strain, and the mutant strain *ecd*^{1} *st ca* (Garen *et al.*, 1977).

Eggs were collected in population cages on trays containing a standard yeast-sucrose medium with chloramphenicol (Nash and Bell, 1968), spread with a paste of live yeast. The wild-type stock was maintained at 25±1°C, while the *ecd*^{1} strain was kept at 20±1°C.

B. Preparation of Larval Epidermis

Approximately 30 grams of late third-instar larvae were spread evenly in a pouch made of nylon mesh (Nitex 390), which was then passed several times between the rollers of a hand-operated wringer. Hemolymph, fat bodies, and other tissues were squeezed out through the pores in the mesh, while the epidermis with overlying cuticle were retained within the pouch. The crude tissue preparation was subsequently collected and rinsed with a dilute solution of PTU* to inhibit phenol oxidase activity. The material prepared in this manner was normally used immediately, but could be quick-frozen in liquid nitrogen and stored at -45°C.

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*For this and other abbreviations, see page xi.

Protein determinations (Lowry et al., 1951) and DDC activity measurements (Clark et al., 1978) were performed on this material to verify that an enrichment for cells producing DDC had been effected over whole larval preparations.

C. Extraction of RNA

All glassware used in the extraction procedure was acid-washed and heat-treated at 150°C for at least four hours to remove RNase. Approximately 15 grams of larval epidermis were ground in 50 ml of extraction buffer (100mM sodium chloride, 2mM magnesium chloride, 1mM EGTA, 0.5% SDS, 10mM Tris-HCl, pH 7.4) in a mortar pre-cooled to -20°C. The homogenate was centrifuged at 12,000xg for 15 minutes, and the supernatant mixed with 2 volumes of phenol-chloroform-isoamyl alcohol (50:49:1). After 15 minutes of gentle agitation, the mixture was centrifuged (4,000xg) at room temperature for 30 minutes. The aqueous layer was removed and re-extracted twice with phenol-chloroform-isoamyl alcohol, followed by one extraction with chloroform-isoamyl alcohol(24:1). The final aqueous layer was brought to 0.2M NaCl, 2 volumes of 95% ethanol were added, and the RNA was precipitated at least 8 hours at -20°C. The RNA was collected by centrifugation, resuspended in buffer (10 mM Tris HCl, 0.05% SDS, pH 7.4), and reprecipitated as above. Finally, the RNA was collected, resuspended, and the absorbance at 260 and 280 nm

determined. The concentration of RNA was calculated on the assumption that a solution of 50 µg/ml had an absorbance of 1.0 at 260 nm. Routinely, yields of 2 mg of RNA per g of tissue were obtained.

D. Oligo(dT)-Cellulose Chromatography

Poly(A)-containing RNA was isolated from bulk larval RNA by chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972). Approximately 0.25 g of oligo(dT)-cellulose (Type 7, P.L. Biochemicals) was equilibrated in application buffer (10mM Tris-HCl, 0.5M NaCl, 0.5% SDS, pH 7.4), and packed into a plastic syringe. The RNA to be applied was made up to 0.5M NaCl and 0.5% SDS, and slowly loaded onto the column at room temperature. The flow rate was kept constant at 12.5 ml/hour with a peristaltic pump. Absorbance was monitored continuously with an Isco UV analyzer and flow-cell, and the column was washed extensively with application buffer until the absorbance returned to baseline. Poly(A)-RNA was released with elution buffer (10 mM Tris-HCl, 0.05% SDS, pH 7.4), and collected into 2 volumes of 95% ethanol containing 150mM sodium acetate (pH 5.5). The RNA was precipitated 8 hours at -20°C, collected by centrifugation (12,000xg, 20 mins, 4°C), resuspended in a small volume of sterile distilled water, and frozen in aliquots at -45°C.

E. Wheat-Germ Cell-Free Translation System

A cell-free protein synthesis system derived from wheat-germ was prepared as described by Roberts and Paterson (1973) with some modifications (Dr. V. Paetkau, pers. comm.). The correct preparation and storage of the extract and translation components was critical in obtaining efficient translation, and therefore is described below in detail.

Storage of Translation Components

Creatine phosphokinase (1mg/ml) was stored at -20°C in 50% glycerol, and was active for at least 4 months. DTT was stored at 4°C. Amino acid mixtures were prepared at 1 mM each, neutralized, and stored at -20°C until use. A mixture of the remaining components in the translation reaction was prepared (see Appendix), and stored frozen in aliquots at -45°C. Once thawed, this reaction cocktail was never refrozen or reused.

Preparation of S-30

All procedures were carried out at 4°C. Four g of wheat-germ (General Mills, Vallejo, Calif.) were ground to a powder in a pre-cooled mortar with 4 g of acid-washed sand. Thirteen ml of extraction buffer (see Appendix) were added, and grinding continued for several minutes. The slurry was centrifuged at 30,000xg for 10 minutes, and 3.5 ml of the supernatant was passed over a column of Sephadex G-25 medium (1.2 x 30cm). The turbid fractions were pooled and recentrifuged at 30,000xg. The final supernatant was divided

into aliquots in 1ml polystyrene centrifuge tubes (Fisher Scientific), and frozen at -45°C.

Extracts prepared in this manner showed no loss in translational activity within 4 months of storage.

Cell-Free Translation

Reactions were carried out in 1ml centrifuge tubes in volumes of 45-270 µl, depending on the nature of the experiment. Reaction components were present in the following concentrations:HEPES-KOH (pH 7.7), 24mM; potassium acetate, 84mM; magnesium acetate, 2.5mM; creatine phosphate, 8mM; ATP (neutralized), 1mM; GTP, 0.2mM;spermidine, 0.48mM; DTT, 2.2mM; creatine phosphokinase, 6µg/ml; wheat-germ S-30, 20%; 20 unlabelled amino acids, 25µM each. ^{35}S-methionine or ^{3}H-leucine were added at concentrations and specific activities described in individual figure legends. Distilled water and poly(A)-RNA were added to the above mixtures as required. It should be noted that the final concentrations of K^{+} ions, Mg^{++} ions and HEPES include the contribution by the S-30 in the reaction. A typical reaction mixture is presented in the Appendix.

Reactions were carried out at 28°C for 2 hours, after which they were immediately placed on ice, and 5µl samples were removed for measurement of total radioactivity incorporated into protein. The remainder was made 10mM in leucine or methionine, and used for immunoprecipitation and analysis on gels.

F. Reticulocyte Lysate Translation System

Preparation of Lysate

New Zealand white rabbits were made anemic by four daily subcutaneous injections of 1ml of 2.5% neutralized phenylhydrazine hydrochloride. They were bled by cardiac puncture on the ninth day after the first injection, using heparin to prevent clotting. The reticulocytes were prepared according to Hunt and Jackson (1974). They were collected by centrifugation (10,000xg, 10 mins, 4°C), washed three times with cold wash solution (130mM NaCl, 5mM KCl, 7.5mM MgCl), and lysed by the addition of 2 volumes of ice-cold water. After centrifugation at 30,000xg for 15 minutes, the lysate was collected and frozen in aliquots at -45°C.

Preparation of mRNA-Dependent Lysate

The reticulocyte lysate was rendered mRNA-dependent prior to use (Pelham and Jackson, 1976). Twenty µl of hemin (1mM, prepared according to Hunt and Jackson, 1973) and 8 µl creatine phosphokinase (5mg/ml in 50% glycerol) were added to 0.78ml lysate before it was completely thawed. Calcium chloride was then added to 1mM, micrococcal nuclease (P.L. Biochemicals) to 10 µg/ml, and the lysate was incubated 15 minutes at 23°C. It was then placed on ice, and EGTA was added to a final concentration of 2mM to inactivate the nuclease. This lysate was then used immediately for protein synthesis.

Protein Synthesis with mRNA-Dependent Lysate

The final translation reaction contained 50%

nuclease-treated lysate, 10mM HEPES-KOH (pH 7.7), 100mM potassium acetate, 0.5mM magnesium acetate, 10mM creatine phosphate, 25µM amino acids (excluding methionine or leucine), and poly(A)-RNA at 15-20µg/ml ^{3}H-leucine or ^{35}S-methionine were added as described in figure legends. The reactions were carried out at 30°C for 60 minutes, after which they were treated in the same manner as the wheat-germ lysates.

G. Radiolabelling of Larval Proteins *In Vivo*

Late third instar larvae were labelled *in vivo* according to the procedure of P.S. Pass (pers. comm.). Fifty mg of wandering third instar larvae (30-35 organisms) were placed in a small glass petri dish in 75µl of 2% ethanol containing 85.6µCi of ^{35}S-methionine (740 Ci/mmol). After 10 hours, crude extracts were prepared by first pressing the larvae between two pieces of filter paper (using the wringer), and then grinding the epidermis in 1 ml of extraction buffer (50mM Tris-HCl, 1mM PTU, ph 7.3 at 22°C). After centrifugation (34,800xg, 10mins, 4°C), the extracts were frozen in aliquots at -45°C.

H. Analysis of Radioactive Proteins

Measurement of Total Incorporation

Total radioactivity incorporated into cell-free translation products and larval proteins was determined by the procedure of Mans and Novelli (1961). Five µl samples

were spotted and dried on squares (1 cm) of Whatman 3MM paper . These were placed in 5% TCA, heated at 90°C for 10 minutes, and rinsed in cold 5% TCA for an additional 10 minutes. The filters were then rinsed in 95% ethanol (5 minutes), ether (5 minutes), and allowed to air dry. Reticulocyte lysate samples were decolourized before drying by rinsing the filters for at least 3 hours in 5% TCA containing 7.5% hydrogen peroxide and 22% formic acid (Woodward et al., 1974). The dried filters were counted in 8 ml of Scintilene (Fisher Scientific) or Aquasol-2 (New England Nuclear) in a Beckman LS-250 liquid scintillation spectrometer. Counting efficiencies were approximately 10% for ^{3}H on filters, and 40% for ^{35}S.

SDS-Polyacrylamide Gel Electrophoresis

Radioactive proteins were analyzed on SDS-polyacrylamide slab gels (10cm x 14cm x 1.5mm thick) using the discontinuous buffer system of Laemmli (1970) with the modifications of Kikuchi and King (1975). A 10% separating gel was used, with a 4.5% stacking gel (30:1 acrylamide-bis). Samples were diluted 1:1 into reducing buffer (3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5mM Tris-HCl, pH 6.8) and boiled for 5 minutes prior to loading. Gels were run at room temperature for 3.5 hours at 90 volts (approx. 12 mamps). They were stained in 0.05% Coomassie R-250 in 10% acetic acid:25%isopropyl alcohol for at least 3 hours, and destained by diffusion in 7% acetic acid.

Radioactive proteins were detected by fluorography

(Bonner and Laskey, 1974) or autoradiography. All gels were dried onto Whatman 3MM paper, and exposed to Kodak RP Royal X-Omat film by pressing the dried gel and film together between two glass plates. Exposures were at 22°C (autoradiography), or -45°C (fluorography) for times indicated in the figure legends.

I. Immunoprecipitation

Purification of IgG

Monospecific antiserum (raised in 12-16 week old male San Juan rabbits) against purified DDC was previously prepared in our laboratory (Clark et al., 1978). Control serum had been collected and prepared from the same rabbits one week prior to the first injection of antigen.

Five ml each of monospecific anti-DDC serum and pre-immune control serum were diluted 1:1 with phosphate-buffered saline (150mM sodium chloride, 10 mM sodium phosphate, pH 7.5), and solid ammonium sulfate was added to 45% of saturation. The precipitates were collected by centrifugation, resuspended in sodium phosphate buffer, pH7.5, and dialyzed overnight against the same buffer. The dialyzed sera were loaded onto columns of DEAE-cellulose (Whatman DE32, 8 ml column volumes), and the first 20 ml of buffer through the columns were collected. These were concentrated to about 6 ml, and protein concentrations were determined. Antibody activity was titrated by incubating various dilutions of the purified sera for 3 hours with a

crude enzyme preparation obtained from young adult flies. The DDC activity remaining after centrifugation (34,800xg, 15mins) was determined.

Preparation of S. aureus Immunoadsorbent

Most strains of Staphylococcus aureus contain a cell-surface protein, protein A, which has a strong affinity for IgG. This property has been exploited in many immunological studies (for review, see Goding, 1978). In the present work, the heat-killed protein A - bearing cells were used as a solid-phase immunoadsorbent.

The staphylococcal protein A - antibody adsorbent was prepared as described by Kessler (1975). A protein A producing strain was grown and maintained on CCY medium (0.5% yeast extract, 0.5% casamino acids, 100mM sodium chloride, 50mM Iris-HCl, pH 7.8) (Arvidson et al., 1971). Ten ml overnight cultures were inocculated into 500 ml broth, and grown at 37°C with shaking for 24 hours. The bacteria were collected by centrifugation at 8000xg, and washed twice with phosphate-buffered saline (pH 7.2) containing 0.05% (w/v) sodium azide (PBS-azide). Cells were resuspended as a 10% suspension in PBS-azide containing 1.5% (v/v) formaldehyde, and stirred for 90 minutes at room temperature. They were then washed, resuspended in PBS-azide in a large erlenmeyer flask, swirled 7.5 minutes in an 80°C water bath, and cooled in an ice-water bath. After one further wash, the cells were made up to a 10% (v/v) solution in PBS-azide and frozen in aliquots at -45°C. Prior to use,

bacteria were collected, incubated 15 minutes in NET buffer (150mM NaCl, 5mM EDTA, 50mM Tris-HCl, 0.02% sodium azide, pH 7.2) containing 0.5% Triton X-100, washed once in NET + 0.05% Triton X-100, and finally resuspended and stored in this same buffer at 4°C for a maximum of 3 weeks.

Immunoprecipitation Reactions

Protein samples from cell-free translation mixtures or labelled larval crude extracts containing 1-12 x 10^6 cpm were diluted 1:1 with NET + 0.5% Triton X-100, and pre-incubated with 10 µl (36 µg) pre-immune IgG for 2 hours at 4°C. Fifty µl of the Staphylococcus aureus suspension (SA) were added, and following incubation for 30 minutes, the cells were removed by centrifugation at 2000xg for 10 minutes. This pre-adsorption step helped to minimize non-specific binding of radioactivity during the subsequent immunoprecipitation. The supernatants were mixed with 25ug anti-DDC IgG, or pre-immune IgG in the case of the control, and incubated at 4°C for 12 hours. Fifty ul of SA were added, and the cells were collected after a 30 minute incubation period. The cells were washed 5 times with NEI + 0.05% Triton X-100, the final suspensions were transferred to fresh tubes, and after centrifugation, the cells were resuspended in 100 µl SDS reducing buffer. These suspensions were boiled for 5 minutes, and cell debris was removed by centrifugation at 4500xg for 15 minutes. The supernatants containing released proteins were loaded directly onto SDS-polyacrylamide gels for analysis.

III. RESULTS

A. Purification of Larval Epidermis for RNA Extraction

In late third instar larvae, most of the dopa decarboxylase activity is found in the epidermal layer (Lunan and Mitchell, 1969). Since DDC makes up only a small fraction of the total protein of the larvae at this time (approx. 0.1%), it was found to be advantageous to purify the starting material for DDC-producing tissues. By passing larvae through a wringer as described in Materials and Methods, a two-fold enrichment for DDC specific activity was obtained. Table 1 shows the results from two separate experiments. In these, one half of a sample of larvae was used as whole organisms, and the epidermis was prepared from the other half. Extracts were prepared from each, and DDC activity and protein concentrations were determined. The variation in specific activities between the two experiments is due to slight differences in the age of the organisms used. Since DDC activity appears very rapidly toward the end of the third instar, a small age difference can result in large enzyme activity differences (M. Estelle, pers. comm.).

This purification procedure had no serious deleterious effects on the epidermal tissues, as they retained dopa decarboxylase activity for several days when cultured *in vitro* in a standard insect tissue culture medium (W.C. Clark, pers. comm.).

Table 1. Enrichment for DDC-producing tissues.

	TISSUE	PROTEIN (mg/ml)	DDC ACTIVITY (units/mg)	PURIFICATION (x)
Expt 1)	Whole	6.6	1.35	2.10
	Epidermis	10.5	2.87	
Expt 2)	Whole	9.0	3.83	2.09
	Epidermis	10.1	8.01	

Epidermis was freed from hemolymph and other tissues as described in Materials and Methods. All extracts were made at 200 mg/ml in 50 mM Tris-HCl, 1mM PTU, pH 7.3 (@ 23^{o}C), and were dialyzed prior to determination of protein concentrations. One unit of enzyme activity corresponds to the decarboxylation of 1 nmole of dopa under the standard assay conditions.

B. RNA Extractions

The phenol-chloroform-isoamyl alcohol procedure for RNA extraction was used throughout the study. This gave consistent yields of RNA which was active in directing protein synthesis in cell-free systems.

RNA extractions were made on two samples of wild-type larvae of different ages. Larvae used as the mid-third instar sample were collected from food trays at a time when some were beginning to wander on the walls of the tray (the wandering stage begins 6-8 hours before pupariation; Hodgetts et al., 1977). At this stage the majority of the larvae were approximately mid-way through the third instar, as determined from a sample of these larvae monitored for pupariation at various times after collection. Figure 1 shows that 50 percent of the larvae had pupariated by 29 hours after collection. Therefore the mean developmental age of the larvae used was slightly younger than mid-third instar (the duration of the third instar is approximately 43 hours at 25±1°C). The rationale for using this larval stage was that since DDC activity just begins to increase at this time, the mRNA must be present. An initial concern was that at later stages when the activity is maximal, the mRNA might already be degraded.

For the late third instar sample, only wandering larvae were used. Care was taken to avoid overcrowding in the food trays, as larvae prematurely leave the food under these conditions.

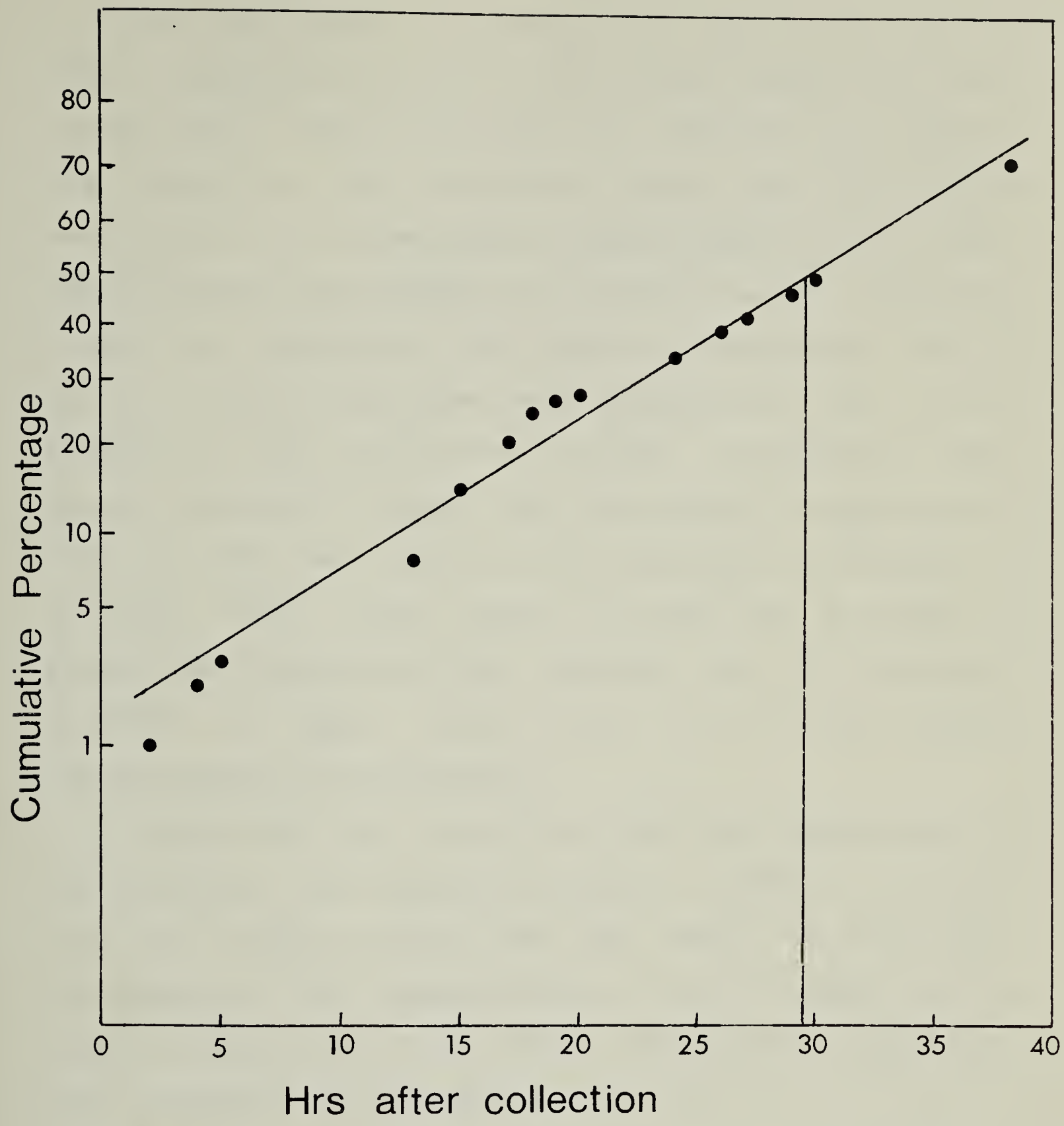


Figure 1. Pupariation profile of wild-type larvae used for RNA extraction.

A sample of the larvae was placed on fresh food, and the total number of prepupae was monitored for 40 hours. Cumulative percentage pupariated is plotted against time after collection (probability graph).

Two RNA samples were prepared from ecd¹ larvae. In one case, larvae were shifted to 29°C mid-way through the third instar. After 3 days, the larvae were collected and used for RNA extraction (low ecdysterone larvae). The other sample was treated in the same manner, except after 3 days at 29°C, 25g of larvae were collected and placed in a tray containing 12.5ml of ecdysterone (500 µg/ml in 2% ethanol). After 8 hours at 23±1°C, the larvae were collected and used for RNA extraction (high ecdysterone larvae). Preliminary small scale experiments showed that DDC activity in ecd¹ larvae kept at 29°C was very low (4.6 units/mg) as compared to wild-type third instar larvae at 29°C (10.7 units/mg). A three-fold increase in DDC activity (to 14.2 units/mg) occurred in ecd¹ larvae after an 8 hour dietary administration of ecdysterone.

Poly(A)-RNA was isolated from bulk RNA preparations by chromatography on oligo(dT)-cellulose. Yields of RNA eluted from the column varied from one sample to the next, depending on the concentration of the RNA loaded and the number of prior uses of the column. Recoveries from the four RNA extractions are presented in Table 2.

C. Characterization of Translation Systems

Wheat-Germ System

The wheat-germ cell-free protein synthesizing system was found to translate Drosophila mRNA efficiently using the conditions described in Materials and Methods. Therefore, no

Table 2. Summarized results of RNA preparations used in cell-free translation and immunoprecipitation studies.

SOURCE (developmental stage)	TOTAL RNA LOADED (mg)*	RNA ELUTED FROM OLIGO(dT)-CELLULOSE (µg)	A260/280
Canton-S (mid-third instar)	26.4	362	2.0
Canton-S (late third instar)	25.3	80	2.0
ecd[1] (late third instar, held at 29°C)	25.0	75	2.1
ecd[1] (as above, but fed 500µg/ml β-ecdysone for 8 hours)	11.6	110	2.1

* Calculated assuming 1 A_{260} unit = 50 µg RNA

further experiments were performed to optimize the concentrations of K+ and Mg++ ions. The final concentration of each were 84mM and 2.5mM respectively.

Figure 2 shows the dose response of the wheat-germ system to added poly(A)-RNA, and Figure 3 shows the time course of protein synthesis at each RNA concentration used. Incorporation is roughly linear with repect to RNA concentration up to 10 µg/ml. Above 20 µg/ml, total incoporation decreases. Addition of optimal levels of RNA typically stimulated incorporation of labelled amino acids 15-25 fold over control levels (no RNA added).

Analysis of cell-free products by SDS-polyacrylamide gel electrophoresis showed labelled proteins migrating up to approximately 70,000 MW, but few higher (see Fig. 6). The subunit molecular weight of dopa decarboxylase is 54,000 (Clark et al., 1978).

mRNA-Dependent Reticulocyte Lysate System

Reticulocyte lysates were rendered mRNA dependent by pre-treatment with micrococcal nuclease, as described by Pelham and Jackson (1976). This procedure digests endogenous lysate (globin) mRNA. After this treatment, background incorporation of labelled amino acids was reduced to approximately 5% of the levels in pretreated lysates (data not shown). Addition of Drosophila poly(A)-RNA to the mRNA-dependent lysates stimulated protein synthesis 5-10 fold, with variation between individual nuclease-treated samples. RNA concentrations were optimal at 20-30 µg/ml

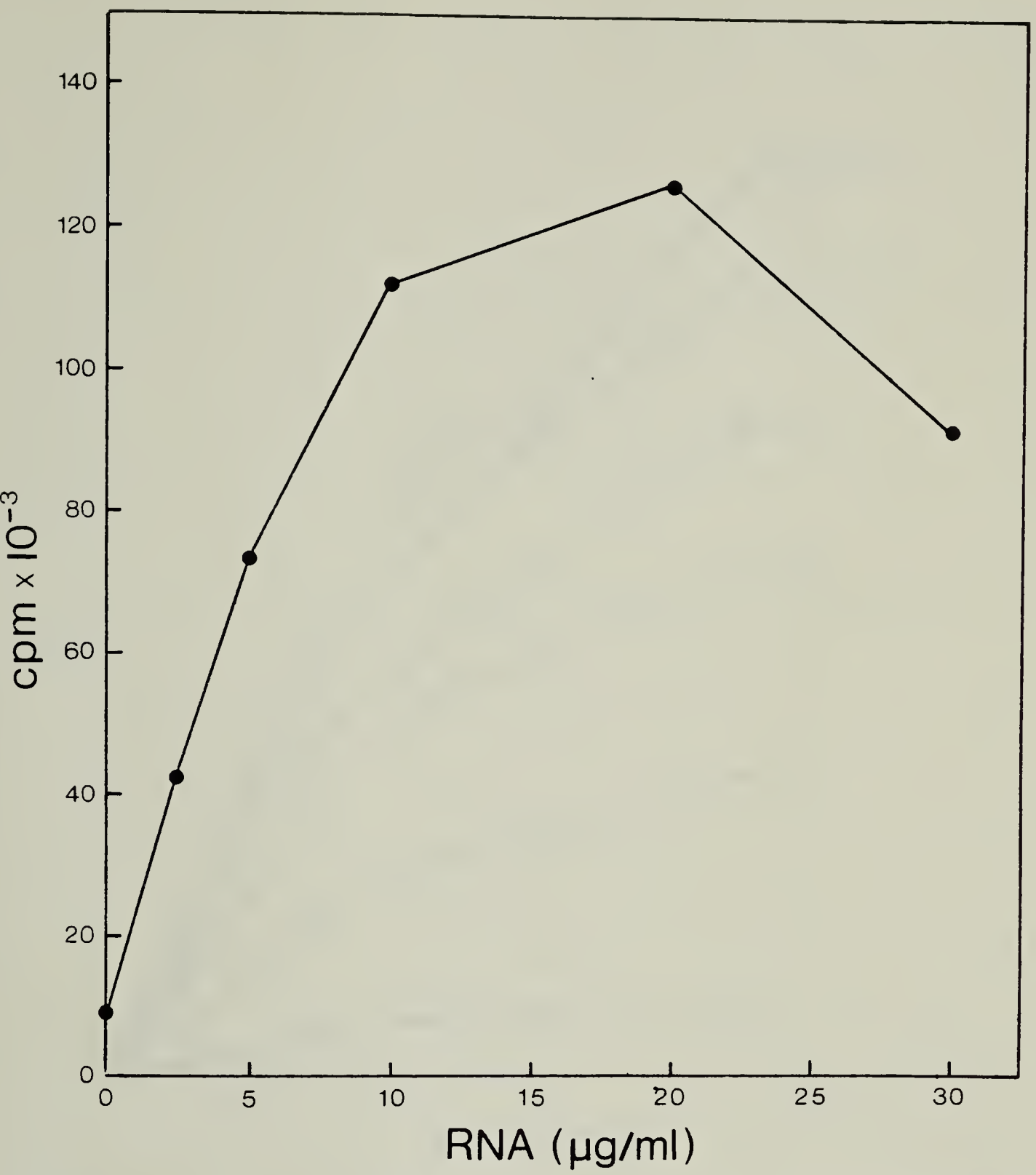


Figure 2. Dose-response of the wheat-germ system to Canton-S mid-third instar poly(A)-RNA.

Standard reactions were set up using ^{3}H-leucine (53 Ci/mmol; 100 µCi/ml) and various concentrations of RNA. Following a 90 minute incubation, 10 µl samples were withdrawn for measurement of hot TCA-precipitable radioactivity. ^{3}H-leucine incorporation is plotted against RNA concentration.

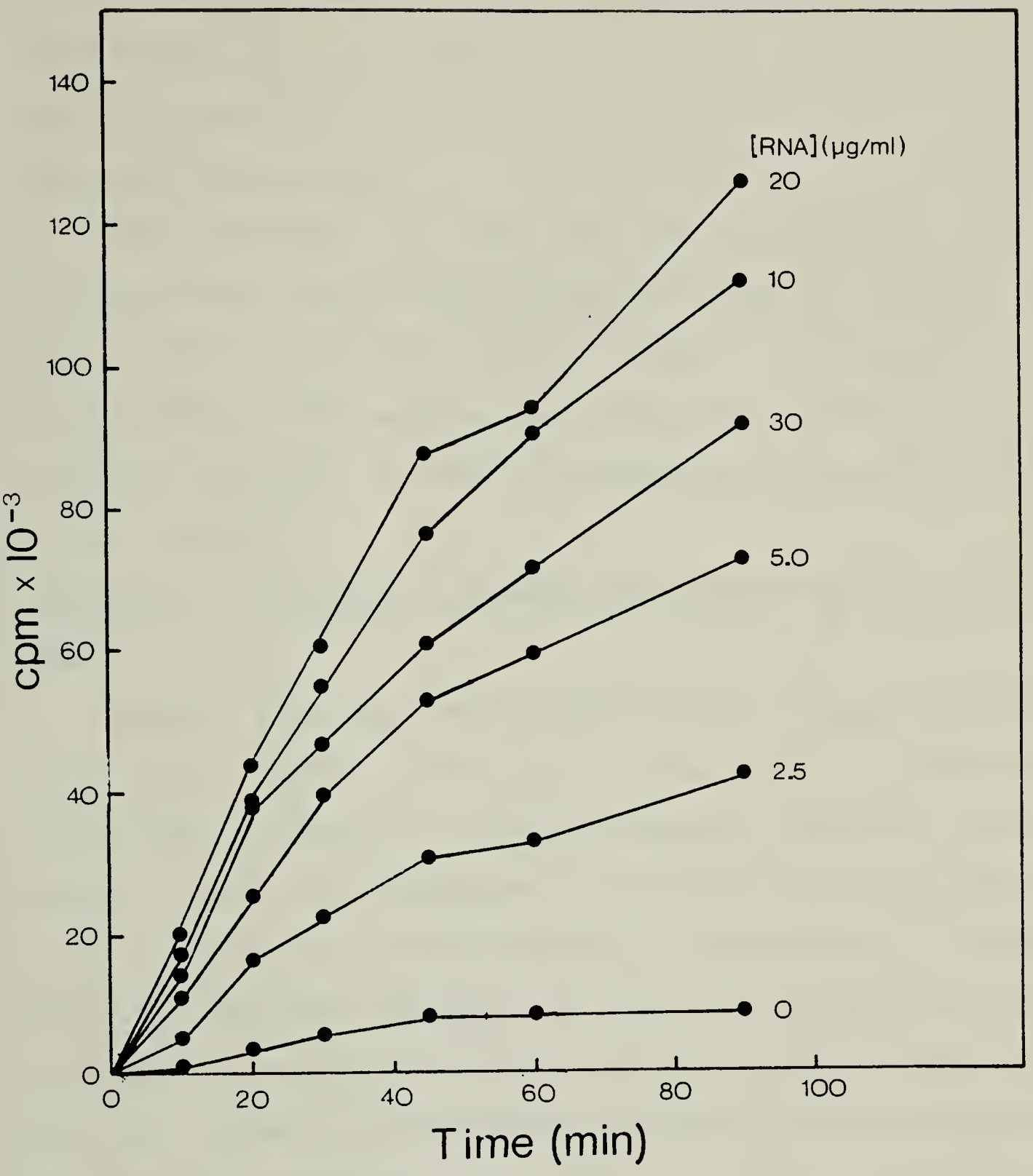


Figure 3. Time-course of protein synthesis in the wheat-germ system.

The reactions described in Figure 2 were assayed for ^{3}H-leucine incorporation in 10 µl samples, at the times indicated.

(Figure 4), and incorporation was complete within 45 minutes incubation at 30°C (Figure 5). Electophoretic analysis of reticulocyte lysate cell-free products revealed proteins well in excess of 70,000 MW (Fig. 6).

Cell-Free Translation

The responses of both the wheat-germ system and the mRNA-dependent reticulocyte system to added poly(A)-RNA from each larval sample are shown in Table 3. Incorporation was approximately the same for all RNA samples except that from mid-third instar C.S. larvae, which was consistently higher in both systems.

Comparison of Larval Proteins Synthesized In Vitro and In Vivo

Larval proteins were radiolabelled in vivo by placing late third instar larvae in a small volume of 2% ethanol containing ^{3}H-leucine or ^{35}S-methionine. Epidermal extracts prepared from these organisms contained (approximately) 7.0 X 10^{6} cpm/ml. An electrophoretic comparison of larval proteins labelled in vivo and cell-free products from both in vitro systems is shown in Figure 6. Many of the larval proteins appear to be translated into full size products in both cell-free systems.

D. Immunoprecipitation Studies

The detection of small quantities of dopa decarboxylase labelled in vivo and in vitro required the use of a sensitive immunological assay. The procedure described in

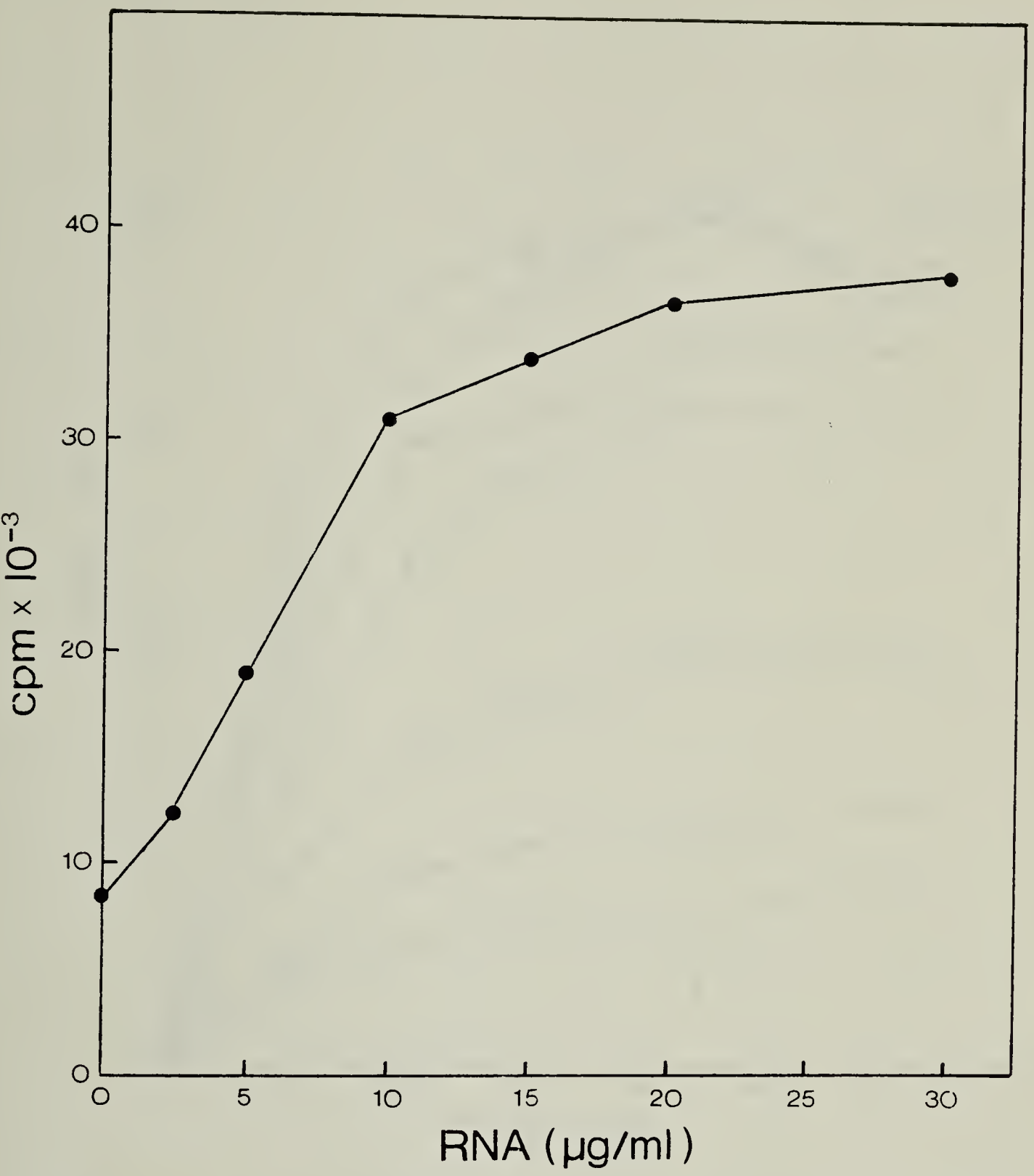


Figure 4. Dose-response of the mRNA-dependent reticulocyte lysate with Canton-S mid-third instar poly(A)-RNA.

Various RNA concentrations were used in standard reactions. Following 60 minute incubations, 10 µl samples were assayed for hot TCA-precipitable radioactivity. Cell-free products were labelled with ^{3}H-leucine (53 Ci/mmol; 200 µCi/ml). ^{3}H-leucine incorporation is plotted against RNA concentration.

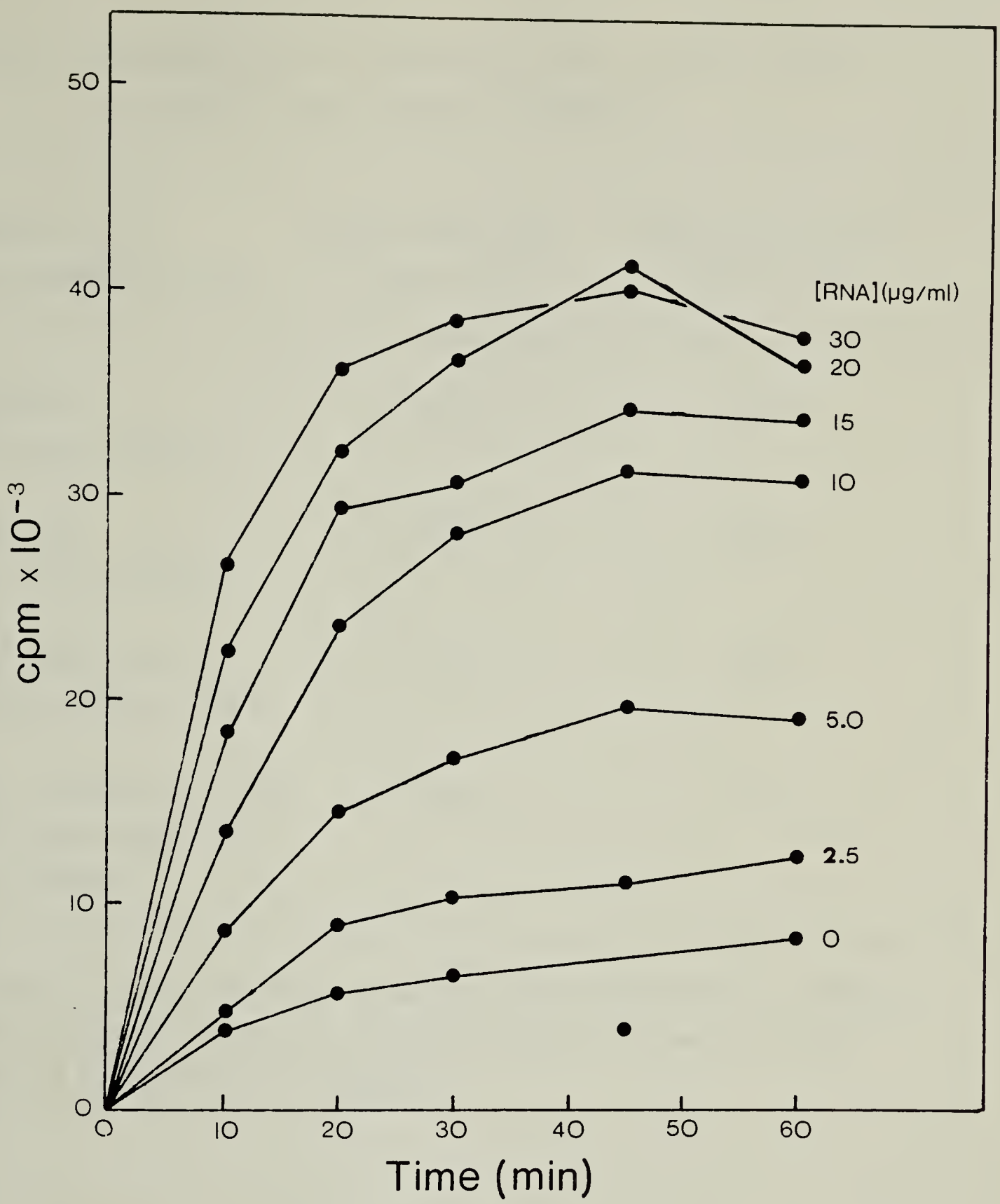


Figure 5. Time-course of protein synthesis in the mRNA-dependent reticulocyte lysate.

The reactions described in Figure 4 were assayed for ^{3}H-leucine incorporation in 10 µl samples at the times indicated.

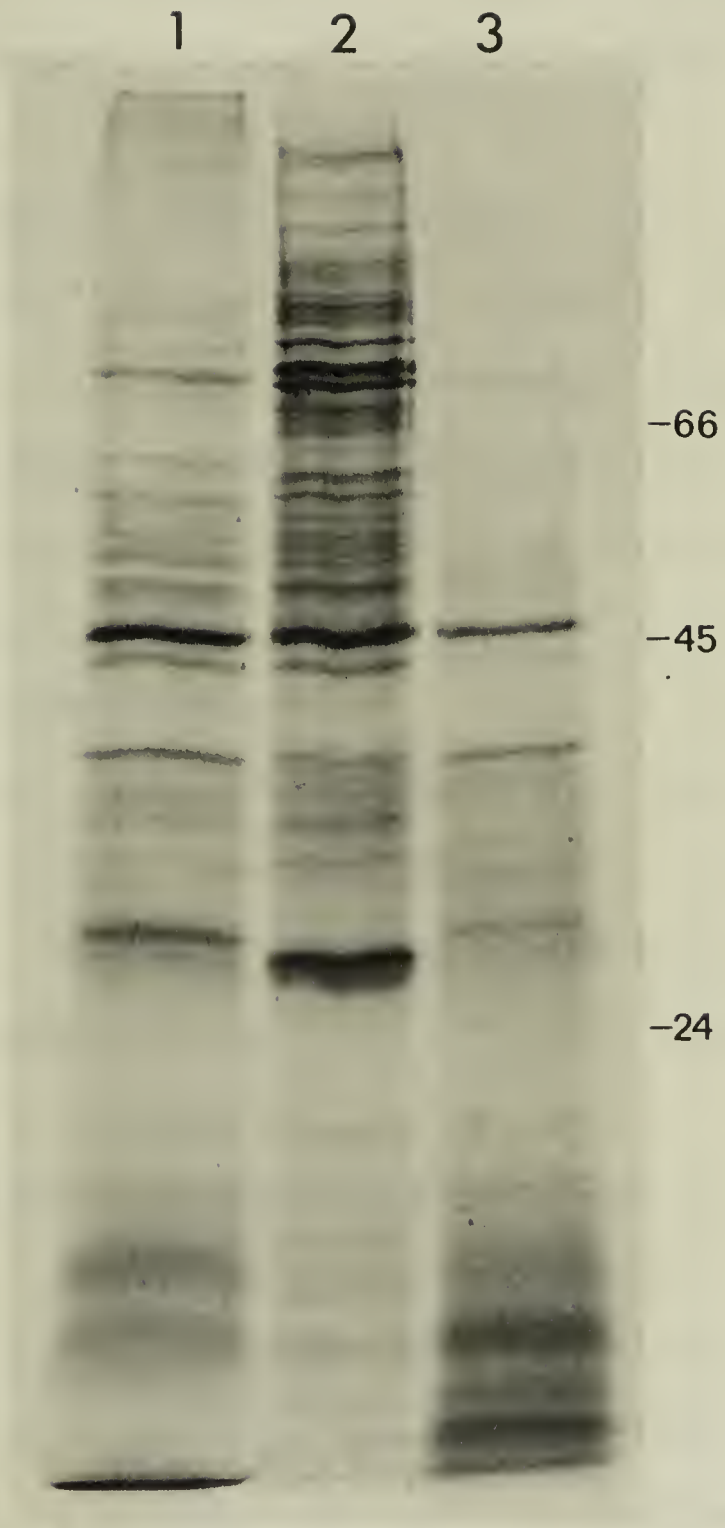
Table 3. Incorporation of ^{35}S-methionine into cell-free products in response to RNA from each preparation.

SOURCE OF RNA (developmental stage)	WHEAT-GERM SYSTEM (cpm x 10^{-4})	RETICULOCYTE SYSTEM (cpm x 10^{-3})
Canton-S (mid-third instar)	58.6	42.9
Canton-S (late third instar)	45.6	22.3
ecd[1] (late third instar, held at 29°C)	39.5	25.7
ecd[1] (as above, but fed 500 µg/ml ecdysterone for 8 hours)	38.6	21.7

Standard reactions were set up as described in Materials and Methods, using 20 µg/ml poly(A)-RNA. Hot TCA-insoluble radioactivity was measured in duplicate 5 µl samples.

Figure 6. Fidelity of translation of larval poly(A)-RNA *in vitro*.

Third instar larval proteins (Canton-S) were labelled *in vivo* with ^{35}S-methionine (740 Ci/mmol). Standard wheat-germ and reticulocyte translation reactions were carried out using larval poly(A)-RNA at 20 μg/ml and ^{35}S-methionine (120 μCi/ml wheat-germ system; 430 μCi/ml, reticulocyte system). Samples were electrophoresed on a SDS-polyacrylamide gel, and radioactive proteins were detected by autoradiography for 4 days. Slot 1 contains reticulocyte lysate translation products; slot 2, proteins labelled *in vivo*; slot 3, wheat-germ translation products. The position of molecular weight markers are shown on the right (MW x 10^{-3}).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this work made use of staphylococcal protein A as an antibody adsorbent. This protein, found on the surface of most strains of Staphyloccus aureus, binds strongly to the Fc portion of IgG molecules --a property which makes it useful in a variety of immunological studies (see Goding, 1978 for review).

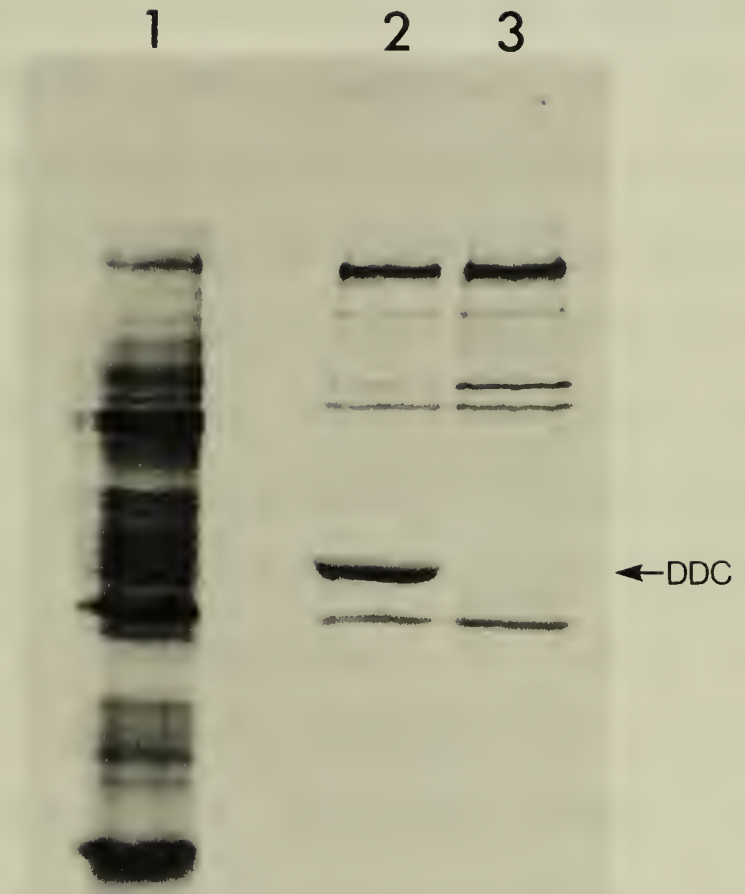
The procedure used involved two steps. First, incubation of the lysates (or extract) with a standard amount of anti-DDC IgG or pre-immune IgG, and second, addition of S.aureus cells to adsorb the IgG molecules. The IgG fractions used were purified from previously collected and characterized sera, as described in Materials and Methods. Immunoprecipitates were analysed by SDS-polyacrylanide gel electrophoresis, and the gels were stained for total protein prior to fluorography or autoradiography. A dark staining band was always seen at 54,000 MW, representing IgG heavy chains released from the immuncadsorbent.

Figure 7 shows the immunoprecipitation of DDC, labelled in vivo with ^{35}S-methionine. In addition to several non-specific proteins, there is a major protein precipitated by anti-DDC IgG, but not pre-immune IgG. This protein comigrates with purified DDC electrophoresed on the same gel.

In an attempt to reduce the non-specific binding of ^{35}S-labelled proteins to IgG, a pre-incubation of all lysates with control IgG was included in subsequent

Figure 7. Immunoprecipitation of DDC labelled *in vivo*.

Canton-S larval proteins were labelled *in vivo* with ^{35}S-methionine. Epidermal extracts were prepared and incubated with anti-DDC IgG or pre-immune IgG, followed by incubation with the staphylococcal immunoadsorbent. IgG-bound proteins were released, electrophoresed on an SDS-polyacrylamide gel, and detected by fluorography of the dried gel for 4 days. Slot 1 contains total proteins labelled *in vivo*; slot 2, proteins bound to anti-DDC IgG; slot 3, proteins bound to pre-immune IgG. The arrow marks the position of purified DDC electrophoresed in the same gel.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experiments. In some cases, several successive pre-incubations of this kind were necessary to reduce non specific adsorbtion to levels which would not interfere with anlaysis of immunoprecipitates on gels (see below).

Figure 8 shows SDS-polyacrylamide gel electrophoresis of total proteins synthesized in the wheat-germ system in response to each RNA sample, and the results of immunoprecipitation from each translation reaction.

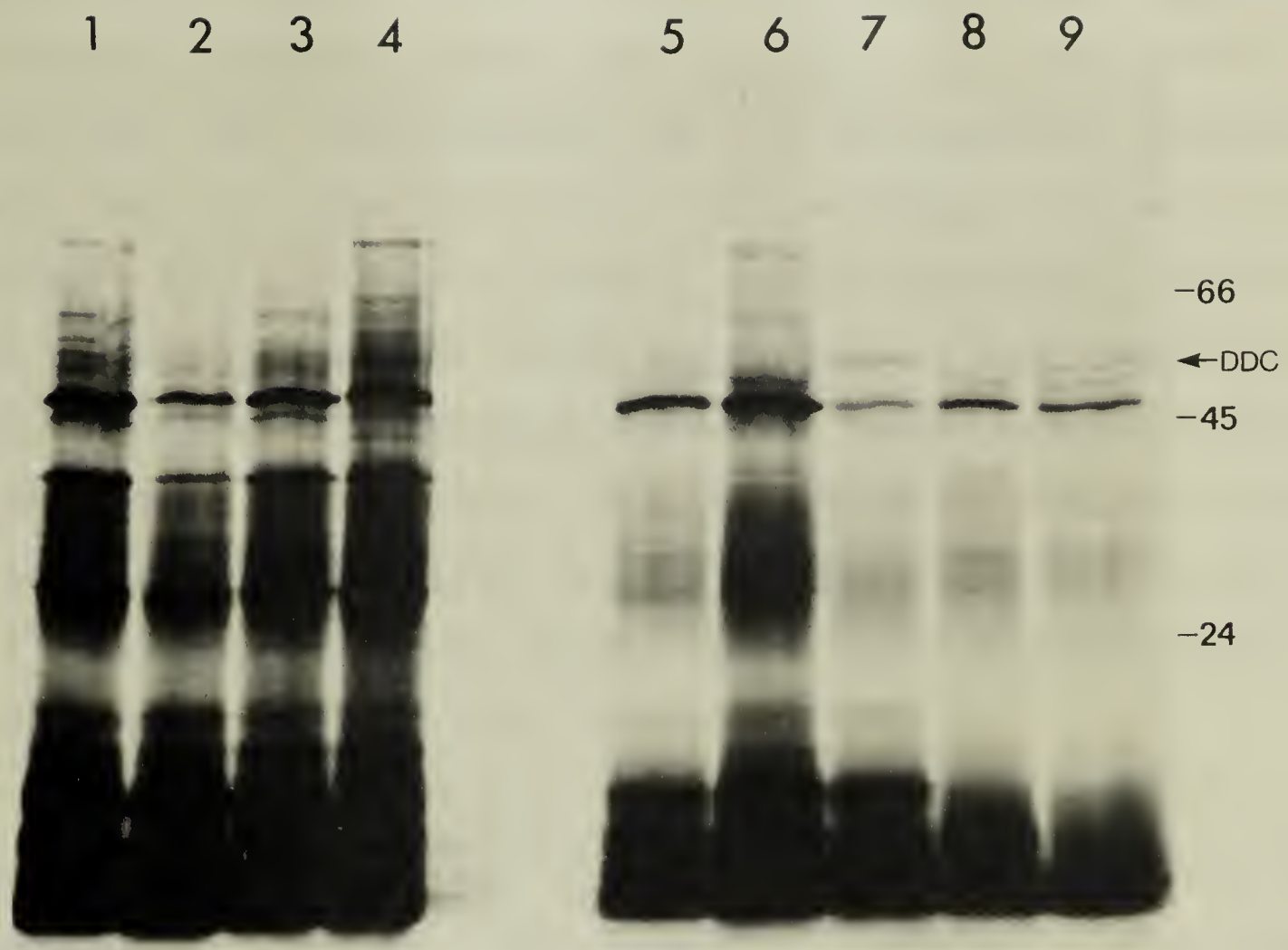
The upper fluorograph in Figure 8 contains the products from wheat-germ translation of RNA from mid-third instar C.S. larvae(slot 1), late third instar C.S. larvae(slot 2), low ecdysterone ecd^1 larvae(slot 3), and high ecdysterone ecd^1 larvae(slot 4). Samples of these cell-free products were treated with either anti-DDC IgG or pre-immune IgG. Slots 5 and 6 show the results of treatment of total translation products of mid-third instar C.S. RNA with anti-DDC IgG and control IgG respectively. There is a faint band present at the same location as marker DDC in slot 5, but not slot 6. This protein is much more evident in immunoprecipitates of translation products of the late third instar C.S. RNA (slot 7) and the high ecdysterone ecd^1 RNA(slot 9). It is completely absent from the low ecdysterone ecd^1 RNA (slot 8).

The lower fluorograph in figure 8 shows the results of a second experiment in which RNAs from late third instar C.S. larvae, low ecdysterone ecd^1 larvae, and high ecdysterone ecd^1 larvae were again translated in the

Figure 8. Wheat-germ translation of larval poly(A)-RNA, and immunoprecipitation of cell-free products.

(Above) Slots 1-4 contain total ^{35}S-labelled proteins synthesized in response to mid-third instar C.S.RNA, late third instar C.S.RNA, low ecdysterone ecd^1 RNA, and high ecdysterone ecd^1 RNA. Slots 5 and 6 show the proteins precipitated from mid-third instar RNA translation products with anti-DDC IgG and pre-immune IgG respectively. Slots 7, 8, and 9 show the proteins immunoprecipitated with anti-DDC IgG from translation products of late third instar C.S.RNA, low ecdysterone ecd^1 RNA, and high ecdysterone ecd^1 RNA. The gel was fluorographed 4 days. The arrow shows the position of purified DDC on the same gel.

(Below) Wheat-germ translation reactions were set up with late third instar C.S.RNA, low ecdysterone ecd^1 RNA, and high ecdysterone ecd^1 RNA. Total proteins synthesized in each reaction were run in slots 1, 2, and 3 respectively. Slots 4, 6, and 8 contain proteins precipitated from C.S. larval proteins, low ecdysterone ecd^1 proteins, and high ecdysterone ecd^1 proteins with anti-DDC IgG. Slots 5, 7, and 9 contain proteins adsorbed from the above samples with pre-immune IgG. The gel was fluorographed for 4 days.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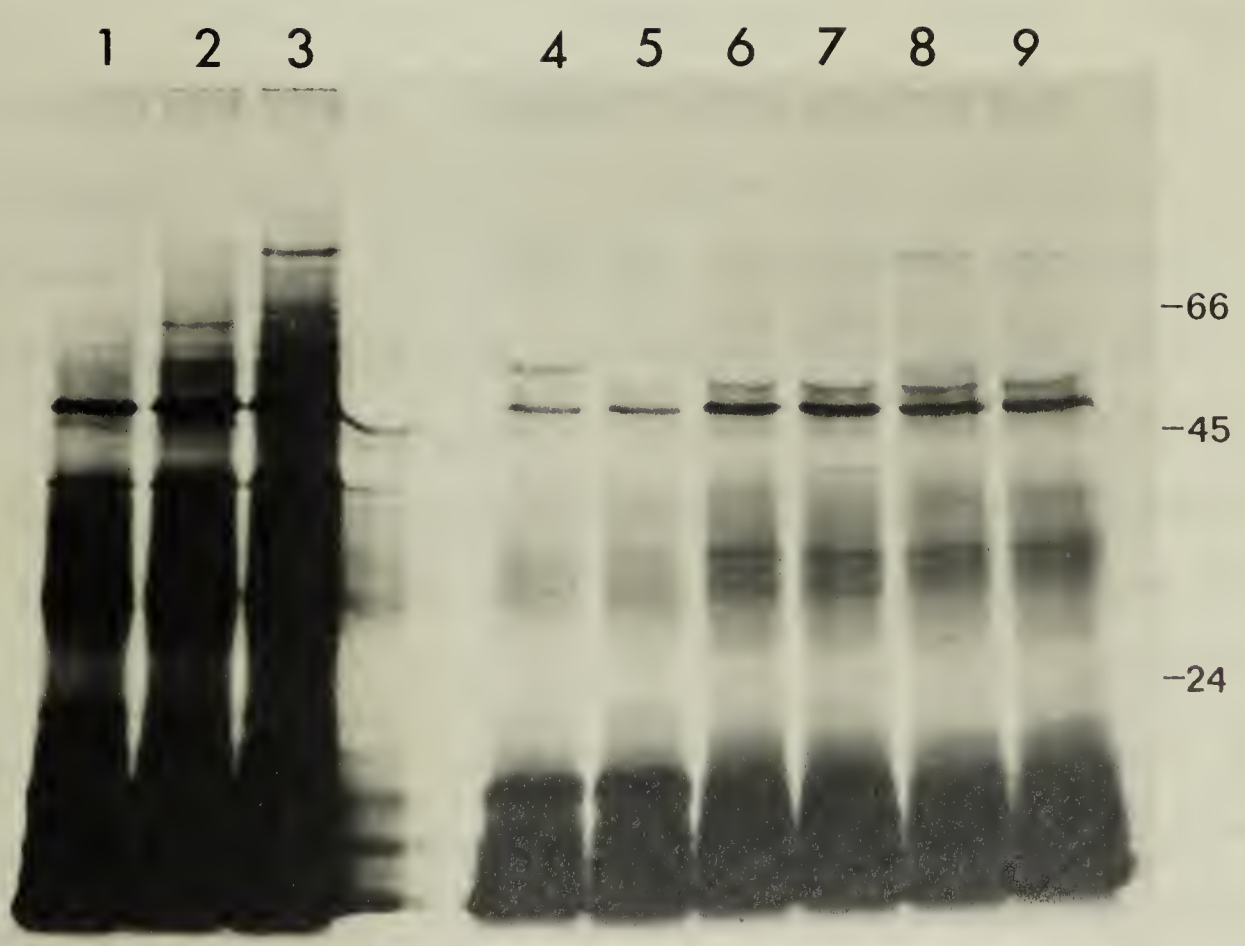
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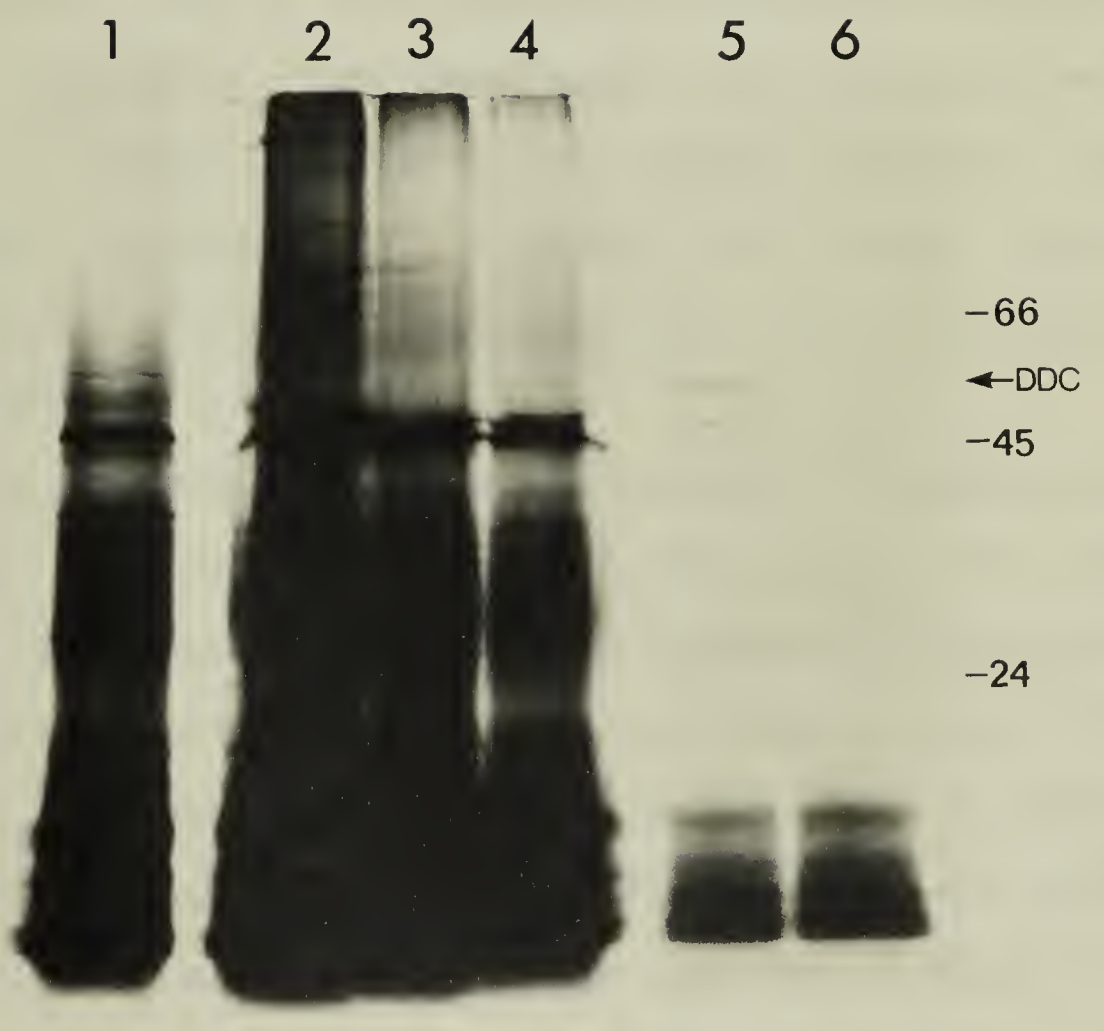
wheat-germ system (slots 1,2, and 3 respectively). Samples of total products from each reaction were treated with anti-DDC IgG and pre-immune IgG, and the reactive proteins were run in slots 4-9. In none of the samples treated with control IgG, is there a labelled protein migrating at 54,000 MW (slots 5,7, and 9). There is, however, a band located at that position in immunoprecipitates from total proteins synthesized in response to wild-type RNA(slot 4) and high ecdysterone ecd^1 RNA(slot 8). The immunoprecipitable protein of this size was not evident in reactions directed by low ecdysterone ecd^1 RNA (slot 6).

As is evident from the fluorographs in Figure 8, non-specific adsorption of radioactive proteins to any IgG molecules tended to obscure results of the immunoprecipitation studies. An experiment was set up to minimize non-specific adsorption, using repeated pre-incubations of lysates with pre-immune IgG. The fluorograph in Figure 9 shows the results of this experiment. Slot 1 contains the total wild-type RNA translation products in the wheat-germ system. Slots 2,3, and 4 show the proteins non-specifically adsorbed to pre-immune IgG after each subsequent incubation. Incubations were carried out at 4°C for 12, 4, and 4 hours respectively, each with 72 µg of pre- immune IgG. The final supernatant, (after removal of control IgG by immunoadsorption to S. aureus cells), was subdivided and incubated with a standard amount of anti-DDC IgG or pre-immune IgG. The proteins

Figure 9. Reduction of non-specific adsorption of radioactive proteins to IgG.

Poly(A)-RNA from wild-type late third instar larvae was translated in the wheat-germ system, and the extract was incubated with pre-immune IgG three times in succession. The final supernatant was then subdivided and treated with anti-DDC IgG or pre-immune IgG. The gel shown was fluorographed for 5 days. Slot 1 contains the total ^{35}S-labelled translation products; slots 2, 3, and 4, non-specifically adsorbed proteins from the three successive pre-incubations; slot 5, proteins immunoprecipitated from the final supernatant with anti-DDC IgG; slot 6, proteins adsorbed from the final supernatant to pre-immune IgG.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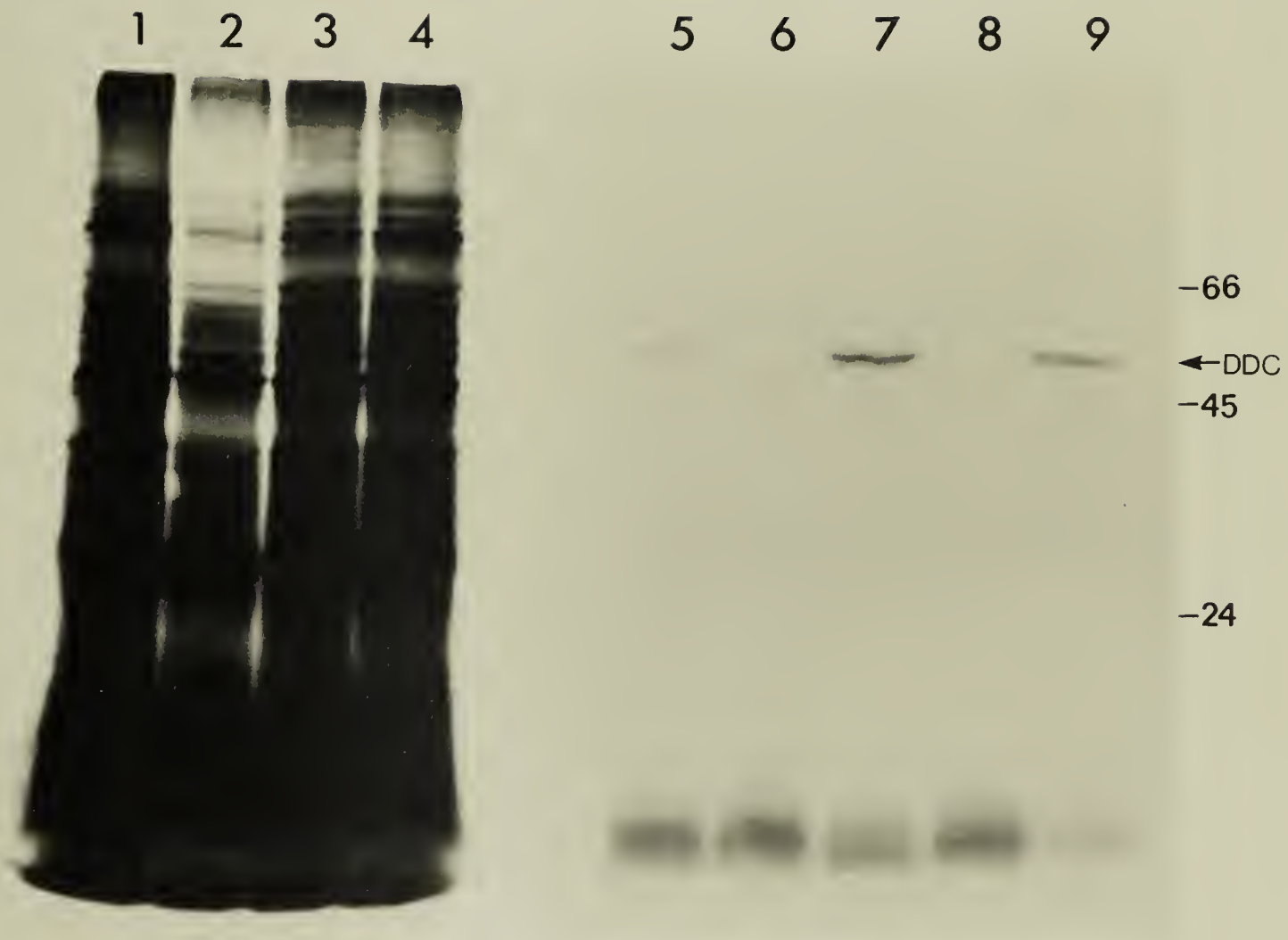
immunoprecipitated with anti-DDC IgG are shown in slot 5, and those adsorbed to control IgG in slot 6. The treatment has clearly reduced non-specific adsorption, as a comparison to Figure 8 shows. Again, an immunospecific protein comigrating with DDC is evident (slot 5).

The fluorograph in Figure 10 shows the results of translation of the four RNA samples in the mRNA-dependent reticulocyte lysate, and immunoprecipitation from the total reaction products. The conditions of the experiment are similar to those described in Figure 8(above) for the wheat-germ system. In contrast to the wheat-germ translation, non-specific adsorption is not a problem in this system.

Slots 1-4 contain the total products of translation of the four RNA samples. Slots 5 and 6 contain the proteins reactive with anti-DDC IgG and pre-immune IgG respectively in the translation products from mid-third C.S. RNA. Slot 7 contains the proteins immunoprecipitated from translation products of late third instar wild-type RNA. The 54,000 MW band is much darker than that in slot 5. Slots 8 and 9 contain the immunoprecipitates from translation of low ecdysterone and high ecdysterone ecd^1 RNA respectively. The specific immunoreactive protein in translation products of both wild-type RNA and high ecdysterone ecd^1 RNA, comigrates with purified DDC electrophoresed on the same gel.

Figure 10. mRNA-dependent reticulocyte translation of larval RNA, and immunoprecipitation of cell-free products.

The gel shown contains: slots 1-4, total ^{35}S-labelled products from translation of mid-third instar C.S. RNA, late third instar C.S. RNA, low ecdysterone ecd^{1} RNA, and high ecdysterone ecd^{1} RNA respectively; slot 5, mid-third instar proteins immunoprecipitated with anti-DDC IgG; slot 6, mid-third instar proteins adsorbed to pre-immune IgG; slots 7, 8, and 9, C.S. late third instar proteins, low ecdysterone ecd^{1} proteins, and high ecdysterone ecd^{1} proteins immunoprecipitated with anti-DDC IgG. The gel was fluorographed for 2 weeks. The migration of purified DDC in the same gel is shown by the arrow.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The results presented above clearly indicate the presence of translatable DDC messenger RNA in poly(A)-RNA from wild-type larvae. Furthermore, this mRNA is identifiable in high ecdysterone ecd^{1} larvae, but not in detectable quantities in ecd^{1} larvae maintained at 29°C.

IV. DISCUSSION

Detection of the primary response to hormone exposure requires both the ability to control the time the target cell is exposed to the hormone, and the ability to quantitate the resulting biochemical changes within the cell. The current model of hormone action suggests the transcription of a specific gene (or genes) to be the initial step in hormone-mediated responses. The approach taken in this study has been the identification of the messenger RNA for dopa decarboxylase, and an analysis of the effect of ecdysterone on the production of this mRNA.

A. Translation and Immunoprecipitation

The availability of monospecific antibody against *Drosophila* DDC made possible the use of cell-free translation to assay for the mRNA coding for this enzyme. Among the accepted criteria for demonstrating the existence of a specific mRNA in a bulk RNA preparation are 1) the translation of this RNA into a product immunoreactive with antibody directed against the purified protein, and 2) the demonstration that the product is identical to the authentic protein by analytical gel electrophoresis or peptide mapping.

The wheat-germ system (Roberts and Paterson, 1973) was first selected for use in this study because of the low endogenous mRNA levels in the extract, and the simplicity and economy of preparing the system. A variety of eukaryotic

mRNAs have been identified using this system, including those for rat preproinsulins (Chan et al. 1976), rat tryptophan 2,3-dioxygenase (Killewich and Fiegelson, 1977), Drosophila histones (Burckhardt and Birnstiel,1978), and rooster vitellogenin (Burns et al., 1978). Here, it translated Drosophila poly(A)-RNA efficiently (Figs 2 and 3; Table 3). Under the conditions described in the Materials and Methods, 9 x 10^7 cpm/ml of ^{35}S-methionine could be incorporated into cell-free products in response to 20 µg/ml poly(A)-RNA. This efficient incorporation allowed several experiments to be performed on a single translation reaction.

Certain disadvantages to the wheat-germ system became apparent during the course of this work. It has a low efficiency of release of nascent peptide chains from ribosomes, and is not normally capable of completing all chains over 50,000 MW (Woo and O'Malley, 1977). This is evident in Figures 6, 8, and 9, where a large proportion of radioactivity can be seen in small peptides, and very few in excess of 70,000 MW. In addition, some RNase activity is found in wheat-germ extracts (Pelham and Jackson,1976).

In light of the above observations, the mRNA-dependent reticulocyte lysate translation system was prepared for comparison. Under the direction of larval poly(A)-RNA, it incorporated radioactive amino acids into proteins (Figs. 4 and 5), but the total incorporation was consistently lower than that of the wheat-germ system (Table 3). This could be

the result of some limiting component in the system. It was not considered serious enough, however, to warrant further analysis. Regardless, the efficient release of nascent chains (Woo and O'Malley, 1977), the synthesis of high molecular weight proteins, and low levels of non-specific binding in immunoprecipation studies have made this system useful in this and other studies (eg. Kourides and Weintraub, 1979; Hunter *et al.*, 1978).

The flucrcgraph in Figure 6 compares the translation of larval poly(A)-RNA in the two cell-free systems. The larval proteins synthesized *in vivo* are, for the most part, synthesized faithfully in both systems. However, unlike the reticulocyte system, the wheat-germ system appears incapable of synthesizing proteins greater than 70,000 MW.

The immunoprecipitation procedure utilizing the staphylococcal protein A-antibody adsorbent has several advantages over the other standard methods. The low concentrations of antigen normally encountered in cell-free translation mixtures prohibit direct precipitation by antibody. One method of circumventing this problem necessitates the addition of "carrier" antigen, to help form precipitable immune complexes. In this study, the availability of purified DDC was limiting.

Another method commonly used involves the addition of a second antibody directed against the first to precipitate all antibody-antigen complexes ("indirect"

immunoprecipitation). The success of this procedure depends on the maintenance of equivalence concentrations between antibodies and antigens, in order to form precipitable complexes. In addition, high backgrounds of non-specific material binding to the complexes are common, and repeated washing of the precipitates results in loss of antigen. Also, proteolytic degradation of the antigen may occur during the long incubations involved in the above procedures (Kessler, 1975 and 1976).

An immunoprecipitation method attempted during the course of this study involved passage of the cell-free translation products over an antibody-Sepharose affinity column to isolate immunospecific proteins. This procedure has reportedly been effective in similar experiments on DDC from Calliphora (Fragouli-Fournogeraki et al., 1978). However, in this study, non-specific adsorption of radioactivity to the column precluded its use as an analytical tool.

In the procedure used in this work, the protein A immunoadsorbent replaces the second antibody in the indirect procedure, and permits the recovery of small amounts of antigen. Since the protein A is fixed to the cell surface, the bound IgG can be recovered simply by sedimentation of the cells, thus eliminating the time necessary for formation of immune complexes. The use of the immunoadsorbent in excess also obviates the need for equivalence point titrations.

The large amounts of radioactivity in the wheat-germ reactions resulted in non-specific binding of radioactive polypeptides to IgG. This was alleviated by successive pre-incubations of translation products with pre-immune IgG (Figure 9).

This immunoprecipitation procedure was used to identify dopa decarboxylase labelled *in vivo*. The experiment shown in Figure 7 illustrates several points. The protein specifically precipitated with anti-DDC IgG (but not with pre-immune IgG) comigrates with purified DDC, thus representing DDC labelled *in vivo*. That DDC could be precipitated out of a crude extract by the procedure described, implied this procedure could be applied to *in vitro* studies as well. The experiment also indicates that larval-protein labelling on a preparative scale could be used to produce enough radioactive DDC for other biochemical analyses (eg. radioimmune assay).

B. Identification of DDC Messenger RNA

The experiments presented in Figures 8, 9, and 10 clearly demonstrate the synthesis of DDC in both translation systems in response to wild-type poly(A)-RNA. It should be noted that the DDC synthesized *in vitro* comigrates almost perfectly with purified enzyme on an SDS-polyacrylamide gel. This suggests that no major post-translational modifications occur in the assembly of the dimeric form of the enzyme *in vivo*.

DDC is labelled more strongly by translation of late larval RNA than mid-third instar RNA, indicating a higher concentration of DDC mRNA molecules to be present at the later developmental stage. It is quite likely, therefore, that this is responsible for the increase in enzyme activity which occurs late in the third instar.

C. Evidence for Hormonal Control of DDC mRNA Production

The increased mRNA level observed in late third instar is correlated with an increased level of ecdysterone (Hodgetts *et al.*, 1977). In order to demonstrate that the hormone is the immediate inducer of DDC, an analysis of the temperature-sensitive *ecd*^{1} mutant was undertaken. The usefulness of temperature-sensitive mutations in *Drosophila* for molecular and developmental studies, has been reviewed by Suzuki *et al.* (1976). The study of ecdysterone-inducible functions is greatly facilitated by the *ecd*^{1} mutation. When raised to 29°C in the third instar, *ecd*^{1} larvae fail to develop the ecdysterone peak normally present at pupariation (Garen *et al.*, 1977), and fail to pupariate. Studies in this laboratory have shown the peak of DDC activity (which normally accompanies the ecdysterone peak) is also absent in these larvae. However, dietary administration of ecdysterone restores enzyme activity (Clark *et al.*, 1979). Mutant larvae retained at 29°C, therefore, have reached a stage at which the presence of ecdysterone is an absolute requirement for further development. Biochemical changes within the larvae

after the administration of ecdysterone may represent specific hormone-induced responses.

The results presented in Figure 10 clearly demonstrate the absence of labelled DDC among proteins translated from low ecdysterone ecd^1 poly(A)-RNA. There is, however, immunoprecipitable DDC synthesized in response to RNA from high ecdysterone ecd^1 larvae. Therefore, the dietary administration of ecdysterone to mutant larvae retained at 29°C (low *in vivo* ecdysterone levels) results in the appearance of translatable DDC messenger RNA within 8 hours.

In light of the current model for hormone action, this observation strongly suggests that the appearance of DDC mRNA activity after hormone exposure results from induced transcription of the DDC structural gene.

There are other alternatives to explain these results. For example, ecdysterone may act to mobilize pre-existing, sequestered mRNA molecules, thus effecting a translational induction. The RNA isolation procedure used in this work, however, would extract all RNAs, whether from polysomes or ribonucleoprotein particles. Thus, one would expect to see DDC mRNA activity in poly(A)-RNA from low ecdysterone ecd^1 larvae.

Another alternative might be post-transcriptional modification --for example, the addition of a "cap" structure at the 5' end or a poly(A) tail at the 3' end of the RNA (Perry, 1976). Still another possibility is that the DDC messenger RNA is synthesized continually, but degraded

rapidly; the presence of ecdysterone could inhibit this degradation, resulting in an increase in the number of mRNA molecules per cell (Tomkins et al., 1972). Although the experiments presented here do not exclude these possibilities, similarities to other hormone systems would suggest a transcriptional induction of DDC mRNA.

First, specific gene transcripts can usually be detected within a few hours of hormone exposure. The DDC mRNA was identified after 8 hours of exposure to ecdysterone. However, the time involved in ingestion of the hormone and transport to the epidermal cells would indicate an even more rapid response. In fact, DDC activity is detectable after only 4 hours of hormone exposure (W.C. Clark, pers. comm.). Since the mRNA activity correlates with enzyme activity during normal development (see above), the amount of translatable DDC mRNA after hormone administration likely increases very rapidly. Second, receptor proteins for several steroid hormones have been identified in target tissues, and these have been shown to interact directly with DNA. While ecdysteroid receptors have not been identified in epidermal tissue from whole organisms, they have been found in imaginal discs (Yund et al., 1978) and the Kc Drosophila cell line (Maroy et al., 1978), both of which consist of ecdysterone-responsive cells. Finally, ecdysterone is known to act directly to induce transcription of specific genes, as indicated by the work of Ashburner et al.(1973). By analogy to other well-characterized hormone systems, these

features would suggest the induction of transcription of the DDC structural gene by ecdysterone to be the most economical and consistent explanation of the results.

To provide direct evidence for transcriptional induction by ecdysterone, a more sensitive assay for RNAs containing structural gene sequences is needed. The development of a translation assay for DDC messenger RNA provides the means for these studies. DNA-mRNA hybrid-arrested translation (Paterson and Roberts, 1977; Hastie and Held, 1978) can be used to isolate synthetic or natural DNA fragments containing the DDC structural gene sequences. This technique has already been used to identify a cloned DNA containing the structural gene sequences for an ecdysterone-induced protein (Lepesant et al., 1978). However, in this case, the function and identity of the protein were unknown.

The cloned DDC structural gene could be used as a probe for specific mRNA sequences throughout development. Analysis of the kinetics of mRNA induction would confirm transcriptional control by ecdysterone. In addition, any relationship of ecdysterone to DDC at other developmental stages could be investigated. During both embryogenesis and the pupal stage, DDC activity peaks occur after prior peaks in ecdysterone titre have already regressed. In embryos, the DDC peak occurs approximately 10 hours after that of ecdysterone (D. Gietz, pers. comm.), while the two are approximately 60 hours apart at the pupal stage (M. Estelle,

pers. comm.). If ecdysterone induces transcription of the DDC structural gene at these stages, the RNA transcripts would have to be stored in a non-translatable form until needed. The cloned DDC gene could be used to determine if this is the case, or indeed, if ecdysterone has any effect at all on DDC regulation at these stages.

Another approach involves the analysis of hormone-receptor binding to chromatin reconstituted from a cloned fragment of DNA. Non-histone proteins from different developmental stages could be tested for their ability to bind ecdysterone-receptor complexes. Studies of this type have been initiated with the ovalbumin system (O'Malley et al.,1977). This system, however, lacks the potential for the genetic analysis available in the study of dopa decarboxylase regulation. For example, studies in progress in this laboratory concern the identification of wild-type strains of Drosophila melanogaster with altered levels of dopa decarboxylase activity (Estelle and Hodgetts, 1979). Cloning the DDC structural gene with adjacent sequences from these strains could be of interest, since the varying levels of enzyme activity may be a consequence of an altered specificity for ecdysterone binding at an adjacent regulatory site.

In summary, the assay for the messenger RNA for dopa decarboxylase has permitted an analysis of the dependence of mRNA production on the presence of ecdysterone during

pupariation. The techniques developed in this study should facilitate further analyses of the regulation of dopa decarboxylase throughout development.

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APPENDIX

Wheat Germ Translation System - Preparation of buffers and reaction mixtures

Materials

Component	Supplier
^{35}S-methionine (600-1200 Ci/mmol)	Amersham, The Radiochemical Center
Creatine phosphokinase, ATP, GTP, creatine phosphate, spermidine, HEPES, DTT	Sigma Chemical Co.
KCl, KOAC, $Mg(OAc)_2$, $CaCl_2$	Fisher Scientific, reagent grade
Sephadex G-25	Pharmacia Fine Chemicals
Wheat germ	General Mills, Inc.

Buffers

Extraction Buffer - 20mM HEPES-KOH (pH 7.7)
100mM KCl
1mM $Mg(OAc)_2$
5mM 2-mercaptoethanol
2mM $CaCl_2$

Column Buffer - 20mM HEPES-KOH (pH 7.7)
120mM KCl
5mM $Mg(OAc)_2$
5mM 2-mercaptoethanol

Reaction Cocktail

53.5µl 20mM ATP (neutralized)
53.5µl 160mM creatine phosphate
10.7µl 20mM GTP
21.4µl 1M HEPES-KOH (pH 7.7)
16µl 100mM $Mg(OAc)_2$
12.8µl 40mM spermidine
32.1µl 2M KOAc
200.0µl - freeze in 50µl aliquots at -45°C

Translation Reaction

50µl reaction cocktail (freshly thawed)
6µl DTT
6.8µl 1mM each of unlabelled amino acids excluding methionine
5µl 0.33mg/ml creatine phosphokinase in 50% glycerol
54µl wheat-germ extract
80-100 µCi ^{35}S-methionine
mRNA, dH_2O to 270µl total volume